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SEROTPING AND MOLECULAR DETECTION OF DIPHASIC AND MONOPHASIC *S. TYPHIMURIUM* ISOLATED FROM FRESH CHICKEN SAMPLES COLLECTED FROM DIFFERENT LOCAL MARKETS IN NAJAF PROVINCE DURING APRIL TO OCTOBER 2012

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ABSTRACT

Salmonella typhimurium is one of most importance serovars associated with human disease reported from poultry in developing countries and during the last few years Monophasic variants of *Salmonella* Typhimurium-like strains, lacking the fljB-encoded second phase H antigen, appear to be of increasing importance in infection in many Member States such as France, Germanyand but research studies in Iraq did not or rare referred to monophasic variants. Therefore a total of 1440 fresh chicken samples were collected from local markets in Najaf city during April to October 2012 to detect typical diphasic and monophasic *S. typhimurium* distribution. The results appeared the numbers and percentages of monophasic *S*. Typhimurium isolates in gizzard, liver and meat were 28 isolates (5.83%), 10 isolates (2.08%) and 3 isolates (0.6%) respectively, when it in typical diphasic *S. typhimurium* raised to 128 isolates (26.66 %), 71 isolates **(**14.79**%)** and 22 isolates (4.5%), respectively, hence the severity of monophasic *S. typhimurium* isolates in this study may be less than it in studying typical diphasic *S. typhimurium* isolates.

KEYWORDS: *S. typhimurium*, Monophasic, Diphasic Chicken Meat

INTRODUCTION

Non-typhoidal salmonellosis (food poisoning) is a food borne disease of primary concern in developed, as well as developing countries. Although this disease is self-limiting, can also lead to life-threatening systemic infections in immunocompromnise patients *((*Feasey *et al*, 2012; Graham, 2010) therefore this disease become among one of the major public health problems that are made worldwide each year up to 1.3 billion cases of acute gastroenteritis and diarrhea resulting in 3 million deaths annually (Razzaque *et al*, 2009; UL-Hassan *et al*, 2008).

S. enterica subspecies *enterica* serovar Typhimurium is considered a major Non-typhoidal *Salmonella* broad host range serovar, usually associated with gastroenteritis in phylogenetically unrelated host species specially associated with human disease worldwide, therefore these are importance to public health commonly reported from poultry in developing countries (;CDC, 2004; Rahman *et al*, 2004, Sharma *et al*, 2005; Archambault *et al*, 2006).

The notation of the antigenic formulas of *S. typhimurium* is

O-Antigens

H-antigens of first phase: H-antigens of second phase, therefore the full antigenic formula of *Salmonella*

typhimurium is: 1, 4 [5], 12:i: 1, 2. Here, the somatic O-antigens are: 1, 4, [5], 12. Where i represent the H-antigens of first phase and 1, 2 is H-antigens of second phase (EFSA**,** 2010; World Assembly of Delegates of the OIE, 2010;).

During the last few years Monophasic variants of *Salmonella* Typhimurium-like strains, lacking the fljB-encoded second phase H antigen, with the antigenic structure 1,4,[5],12:i:- appear to be of increasing importance in many Member States such as France, Germany, Austria, Ireland, Italy, Denmark, the Netherlands and Luxembourg. Such variants are referred to as 'monophasic *S.* Typhimurium'. Strains lacking expression of the phase one or both flagellar antigens are also possible, but uncommon to be associated with significant disease in animals or humans. Therefore, for the purposes of this Opinion, only the monophasic variants lacking second phase H antigens were considered. (EFSA, 2010; World Assembly of Delegates of the OIE, 2010). Research studies in Iraq did not or rare referred to this diphasic and monophasic variants therefore the present was aimed to differentiate the typical diphasic and monophasic *S. typhimurium* distribution in locally freshly chicken samples in Najaf province.

MATERIALS AND METHODS

Collection of Chicken Sample (Study Area and Period)

The research was focused on Najaf province. The study population included only the fresh chickens (that use as human food) from 15 different locally markets regions that distribute in Najaf during the period extended from April to October 2012, a total of 480 chicken were taken from four locally markets randomly in each region as 8 chicken (weighed 1-1.5 Kg) from each market, putted into separate plastic bags, cooled in an ice box and immediately transported to laboratory. The different samples of each chicken (liver, gizzard and meat) collected individually, homogenized and diluted.

Isolation and Identification of *S. typhimurium*

Twenty five grams from each Meat, Liver and gizzard of each chicken were individually homogenized and diluted in 225ml of Tetrathonat broth medium then from each one of sample, serial dilution were performed to10^{-} . One milliliter from each dilution was cultured on selective enrichment XLD medium in three replicates, incubation at 43°C for 24 h. for isolation of enteric pathogens *Salmonella*, and incubation was extended to 48h to increase visibility of H_2S Production. **(**Wallace *et al*, 2011).

Calculate the Percentage of Typical Diphasic and Monophasic *S. typhimurium* Isolates

The presumptively positive *Salmonella* isolates were confirmed by the biochemical method **(**Wallace *et al*, 2011) and *S. typhimurium* with slide agglutination test and Multipluex PCR assay and then the percentage of typical diphasic and monophasic of *S. typhimurium* isolates Calculated by equation below (Ali, 2007)

$$\text{Percentage of Isolates} = \frac{\text{Number of isolates}}{\text{Total number of collected samples}} \times 100$$

Serological Test

All isolates identified biochemically as *Slamonella* were serologically examined by a slide agglutination test that performed with Polyvalent "O" Antisera, specific O antisera (SIFIN), Polyvalent H antisera And specific H antisera (SIFIN), The somatic (O) and flagellar (H) antigens were characterized by slide agglutination with commercially available anti-sera (SIFIN, Germany) and the serotype was assigned according to the Kauffmann-White scheme.

(Wallace *et al*, 2011; Saraj and Stefan. 2009).

Molecular Differentiation of a Typical Diphasic *S. Tphimurium* and Monophasic 4, [5], 12: I

In order to differentiate *Salmonella* strains belonging to the somatic antigen group "B" and sharing the same first flagellar antigen 'i', a multiplex PCR used to support the serological identification of the antigens. (Tennant *et al*, 2010).

DNA Extraction

DNA Extraction was performed depending on method mentioned by Tennant *et al*, (2010).

- A strain of *Salmonella* spp. was spreaded on a non selective agar tube
- The tube incubated at 37°C ± 1°C for 18-24 hours
- four hundred µls of nuclease free water were added in a 1,5ml or 2ml dnase free tube and suspend the bacteria
- The bacterial cells mixed (e.g. using a vortex)
- Absorbance was reared at 600nm. Correct absorbance to be comprised between 1 and 2.
- The tube Incubated at 100°C for 15min
- Centrifuge at 20°C to 25°C for 5 min. at 10,000g
- The supernatant collected and stored at -20°C

Multipluex PCR Assay Primers

The list of primers used to genes for identification and differentiation of diphasic *S. typhimurium* and monophasic 4,[5],12:I**:-**using multipluex PCR assay were (BioNeer). These primers are given in Table 1.

Table 1: Primer Sequence of the Genes for Differentiation of Diphasic *S. typhimurium* and Monophasic 4, [5], 12: I:-Using Multipluex PCR Assay

Primer	Target Gene	Primer Orient-Ation	Sequence (5—3-)	Amplification Product (Bp)	Reference
Primer FFLIB	Flic *fliC*	F	5-CTGGCGACGATCTGTCGATG-3	1000 bp	(Tennant *et al.*, 2010, (EFSA,2010)
Primer RFLIA		R	5-GCGGTATACAGTGAATTCAC-3		
PrimerSense-59	*fljB* allele	F	5-CAACAACAACCTGCAGCGTGTGCG-3	1389 bp	(Tennant *et al.*, 2010, (EFSA,2010)
Primer Antisense-83		R	5-GCCATATTTCAGCCTCTCGCCCG-3		

Preparing the Primers

The Bioneer primers were prepared depending on manufacturer instructions by dissolving the lyophilized product with TE buffer molecular grad after spinning down briefly. Working primer tube was prepared by diluted with TE buffer molecular grad. The final picomoles depended on the procedure of each primer.

A Multiplex PCR Cycling Profiles

A multiplex Polymerase chain reaction assays were carried out in a 20 µl reaction volume, and the a multiplex PCR amplification conditions performed with a thermal cycler were specific to each single primer set. (Tennant *et al*, 2010). (Table 2) represents the amplification conditions depending on their reference procedure.

Table 2: The Multiplex PCR Amplification Conditions of Identification and Differentiation of Typical Diphasic *S. Tphimurium* and Monophasic 4,[5],12:I:

All Multiplex PCR Programs Consisted of 35 Cycles						
Gene Name	Primer	Initial Denaturatio / Time	Denaturation / Time	Annealing / Time	Extension / Time	Elongation / Time
fliB-fliA intergenic region of the flagellin gene cluster included fliC	FFLIB RFLIA	95/2min	90/30 sec.	64°C/30sec	72/1min	72/10min
fljB allele	Sense-59 Antisense-83	95/2min	90/30sec.	64°C/30sec	72/1min	72/10min

Preparation of Agarose Gel

Agarose gel was prepared by adding 1 gm of agarose powder to 100 ml of TBE buffer previously prepared (90 ml D.W were added to 10 ml TBE buffer l0X) in conical flask, the final concentration was 1 X and pH 8. The conical flask was placed in boiling water bath until it become clear and then allowed to cool to 50°C, and 1.5 µl ethidium bromide at concentration of 0.5 mg/ml was added. The agarose poured kindly in equilibrated gel tray earlier set with two combs fixed in the end and in the middle, and the two ends of gel tray were sealed. The agarose allowed solidifying at room temperature for 30 minutes. The comb was removed gently from the tray and the seal was removed from the ends of the tray. The comb made wells used for loading DNA samples.

Multiplex PCR Product Analysis

Agarose Gel Electrophoresis

The amplified PCR products were detected by agarose gel electrophoresis and visualized by staining with ethidium bromide. PCR products were loaded to the agarose gel wells: 5µl from single product to single well in known sequence, followed by 100 bp ladder to one of the wells in each row. The gel tray was fixed in electrophoresis chamber. IX TBE buffer was added to the chamber until covering the surface of the gel. The electric current was performed at 60 volt for 1.5 hour.

Electrophoresis Results

The electrophoresis result was detected by using gel documentation. The base pair of DNA bands were measured according to the ladder. The positive results were distinguished when there was DNA band equal to the target product size. Finally, the gel was photographed using gel documentation saving picture

Statistical Analysis

The data were analyzed statistically, using the least significance differences test (LSD), in experiment of one factor and analysis of variance (ANOVA) at the P value level of 0.05 (Daniel, 1999).

RESULTS AND DISCUSSIONS

Serological Characteristics of *S. Typhimurium*

In order to confirm the identification of *Salmonella* isolates and for obtain the serovar Typhimurium**,** serological examination was done for isolates which represented biochemical test of *Salmonella*, there are only 369/662 (55.7%) isolates were appeared positive agglutination results with Anti-salmo. Gr B serra and Anti-salmo. poly-H 1&2 sera where 262/662 (39.2%) of isolates gave positive results with Anti-*Salmonella* Hi sera which represent phase 1 and were 221/662 (33.3%) appear agglutination with Anti-salmonella H2 sera which represent phase 2, Table 3.

Table 3: Frequency of *S. typhimurium* Serotyping in Liver, Meat and Gizzard of Chicken Samples

Sample	Total No. of suspected *Salmonella* Colony	Frequency.(%) of Positive Isolates in Each Serotyping Test						
		Anti-salmo. Gr B	Anti-salmo. O5	Anti-salmo. O4	Anti-salmo. Poly-H	Anti-salmo. Hi (phase1)	Anti-salmo. H2 (phase2)	Nolmalsalin (Control)
Liver	220	98/220 (44.5%)	98/220 (44.5%)	98/220 (44.5%)	98/220 (44.5%)	81/220 (36.8%)	71/220 (32.2%)	0
Meat	75	38/75 (50.6%)	38/75 (50.6%)	38/75 (50.6%)	38/75 (50.6%)	25/75 (33.3%)	22/75 (29.3%)	0
Gizzard	367	233/367 (63.4%)	233/ 367 (%)	233/ 367(%)	233/ 367 (%)	156/367 (42.5%)	128/367 (34.8%)	0
Total	**662**	**369/662 (55.7%)**	**369/662 (55.7%)**	**369/662 (55.7%)**	**369/662 (55.7%)**	**262/662 (39.2%)**	**221/662 (33.3%)**	**0**

The final results in Anti-*Salmonella* Hi (phase1) and Anti- *Salmonella* H2(phase2) experiments indicated that total *S. typhimurium* isolates were either 262 isolates or 221 isolates because the final test to indicate serovars of *S. typhimurium* depend on results of this two tests(EFSA, 2010) but the difference between number of *S. typhimurium* isolates in this two serotyping tests may be related to lacking of fljB-encoded second phase H antigen, (Hopkins *et al*, 2010, Alcaine *et al*, 2006) or to different mutations and deletions have been associated with the lack of phase 2 flagella expression genes (Garaizar *et al*, 2002) .

Molecular Study for Identification and Differentiation of Typical Diphasic and Monophasic *S. Typhimurium*

However serological test indicated *S. typhimurium* isolates were 262 isolates which appeared first flagellar phase but 221 isolates from it do not appeared flagellar phase 2**.** So in order to differentiate *S. typhimurium* strains belonging to the somatic antigen group "B" (group that contain *S. typhimurium* serovar) and sharing the same first flagellar antigen 'i', a multiplex PCR used to support the serological identification of the flagellar antigens. This test appeared 262 isolates (100%) have first flagellar antigen 'i' gene but second flagellar antigen gene (*fljB* allele) that encoded phase 2 '1,2' was positive only in 221 isolates 221/262(84.35%),(figure 1, table 4).

Table 4: Result of Multiplex PCR for *Salmonella typhimurium* Isolates

Gene	Serotyping Phase	Number of Isolate %	
		Positive	Negative
fliB-fliA intergenic region of the flagellin gene cluster	Phase 1	262(100)	0
flj B	Phase 2	221(84.35%)	41(15.64%)

Depending on data mentioned above the Monophasic variants of *Salmonella* Typhimurium-like strains, lacking the fljB-encoded second phase H antigen appeared only in 41isolates 41/262(15.64%)(5).

The lacking of fljB-encoded second phase H antigen which characterized Monophasic *S.* Typhimurium- Like strains may be related to mutations and deletions in fljB-encoded second phase H antigen. There are some investigators reported such mutations and deletions leading to this resultes, Garaizar *et al.*(2002) found at least some of the *S.* 4,5,12, i:– isolates from Spain appear to be characterized by deletion of a large fragment, including *flj*B and *hin*, encoding a DNA invertase essential for *flj*B expression. Where some researches referred to lacking fljB-encoded second phase H antigen, (Hopkins *et al*, 2010; Alcaine *et al*, 2006)**.**

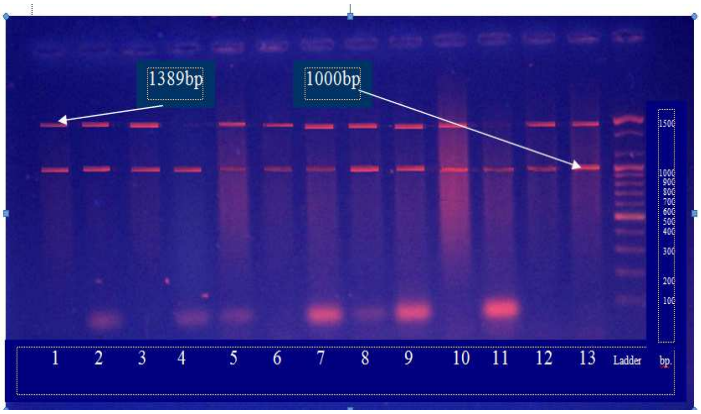

Figure 1: Ethidium Bromide- Stained Agarose Gel of Multiplex PCR Amplified Products from DNA of Some *S. Typhimurium* Isolates Extracted that Amplified With Primers FFLIB(F) and RFLIA(R) (Encoded Flib-Flia Intergenic Region of the Flagellin Gene Cluster) and Sense-59(F) and Antisense-83(R) (Encoded *Fljb* Allele). Lane (Lad) Show DNA Marker (100 Bp). Lane (1-3), (5-10) and Lane(12-13) Show Positive Results with both Flib-Flia Intergenic Region And *Fljb* Allele While Lane(4) And (11) Show Positive Results Only with Flib-Flia Intergenic Region and Nagative Results with *Fljb* Allele

Numbers and Percentage of Typical Diphasic *S. Typhimurium* and Monophasic Variants of *S.* Typhimurium-Like Strains in Fresh Chicken Food Samples

The numbers and percentages of monophasic *S.* Typhimurium isolates in gizzard, liver and meat were 28 isolates (5.83%), 10 isolates (2.08%) and 3 isolates (0.6%) respectively, when it in typical diphasic *S. typhimurium* raised 128 isolates (26.66 %), 71 isolates **(**14.79**%)** and 22 isolates (4.5%), respectively (table 5).

Table 5: Number and Percentage of Typical Diphasic *S. typhimurium* and Monophasic Variants of *Salmonella* Typhimurium-Like Strains in Liver, Meat and Gizzard of Fresh Chicken Food Source

Food Sample of Chicken Source	Number of Examined Sample	Total Number of *S. typhimurium* Isolate	Number & Percentage(%)	
			Typical Diphasic *S.* Typhimurium	Monophasic *S.* Typhimurium- Like Strains
Liver	480	81	71 (14.79%)	10(2.08%)
Meat	480	25	22 (4.5%)	3(0.62%)
Gizzard	480	156	128 (26.66 %)	28 (5.83%)
Total	**1440**	**343**	**221 (15.34%)**	**41(2.84%)**
LSD P≤ 0.05			8.3	2.1

The data reported to ECDC do not suggest infections with the monophasic variants to be more severe. This conclusion is based on the percentage of strains isolated from blood compared to faeces, as this ratio was in the same range or even lower, than for other *S.* Typhimurium (which had 1.8% isolated from blood and 95% from faeces). (EFSA, 2010); therefore, this part of our study investigated of the number and percentage of monophasic *S.* Typhimurium isolates compared with that's of typical *S.* Typhimurium.

As regard the data in Table 5 the severity of monophasic *S.* Typhimurium isolates in this study may be less than it in studying typical diphasic *S. typhimurium* isolates depend on (EFSA, 2010).

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